AD_____

Award Number: W81XWH-07-1-0023

TITLE: Role of GGAP/PIKE-A in prostate cancer progression

PRINCIPAL INVESTIGATOR: Michael M. Ittmann M.D., Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
                           Houston, Texas 77030

REPORT DATE: May 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
14-05-2008	Annual	15 APR 2007 - 14 APR 2008

4. TITLE AND SUBTITLE
Role of GGAP/PIKE-A In Prostate Cancer Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0023

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Michael M. Ittmann M.D., Ph.D.

Email: mittmann@bcm.tmc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, Texas 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The clinical behavior of prostate cancer is extremely heterogeneous, ranging from indolent disease to aggressive, metastatic cancer with rapid mortality. GGAP2 is a GTPase protein with a GTPase activating protein (GAP) domain at the C-terminus. It was reported that GGAP2, or PIKE-A, can activate Akt and inhibit apoptosis. The goal of this proposal is characterize the role of GGAP2 in prostate cancer progression. Our results indicate that GGAP2 expression is increased in prostate cancer. In addition, GGAP2 is mutated in human prostate cancer. The multiplicity of GGAP2 mutations imply that these mutations may emerge in an environment of the multifocal heterogeneity of prostate cancer tissues. According to our results these mutations can stimulate cancer cell proliferation and enhance GGAP2 activation, indicating that they may contribute to increased activity of GGAP2 in prostate cancer. In summary, GGAP2 may promote prostate cancer growth and progression via overexpression and/or mutation and as such is an important therapeutic target.

15. SUBJECT TERMS
GGAP2, Akt, mutation, progression

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION

Prostate cancer is the most common visceral malignancy in US men and the second leading cause of cancer deaths. The clinical behavior of prostate cancer is extremely heterogeneous, ranging from indolent disease to aggressive, metastatic cancer with rapid mortality. GGAP2 is a GTPase protein with a GTPase activating protein (GAP) domain at the C-terminus (Xia et al., 2003). It was reported that GGAP2, or PIKE-A, can activate Akt and inhibit apoptosis (Ahn et al., 2004). The goal of this proposal is characterize the role of GGAP2 in prostate cancer progression.

BODY

As outlined in our Statement of Work, we will seek to accomplish four major tasks in our three years of funding. We have accomplished or made substantial progress on all of these tasks.

Task 1: (Months 1-12) Immunohistochemical studies of GGAP2 in prostate cancer

We have initiated studies of GGAP2 expression in prostate cancer. To examine whether GGAP2 was overexpressed in prostate cancer we performed Western blots of 8 prostate cancers and 8 normal peripheral zone tissues, which revealed expression of GGAP2 in 3 of 8 cancers but in none of the benign tissues (Fig. 1A). The antibody used also detects PIKE-L since it recognizes a carboxy-terminal epitope, but no evidence of PIKE-L expression, which would show a 125 kDa protein band, was seen and only the 90kDa GGAP2 protein was present in the Western blot. We have carried out immunohistochemistry with anti-GGAP2 antibody using tissue microarrays containing matched normal, cancer and high-grade prostatic intraepithelial neoplasia (PIN) tissues. Moderate to high expression of GGAP2 protein was seen in 75% of prostate cancer tissues within the cancer epithelial cells. Staining was primarily cytoplasmic (Fig. 1B) in those cases with staining. Nuclear staining was also identified in some cases (Fig 1C). Similar staining was observed in high grade prostatic intraepithelial neoplasia (Fig. 1D). Absent or weak staining was seen in the more than 95% of the normal epithelial tissues (Fig. 1E). Weak staining of stromal tissues was also noted. We then quantified cytoplasmic expression of GGAP2 using a visual quantitation as described previously (Agoulnik et al 2006). This quantitation is based on a multiplicative score based the extent (scored 1-3) and intensity (scored 0-3) of staining and results in scores ranging from 0 (absent) to 9 (strong, diffuse staining). Results are summarized in Figure 1F. The mean score for normal epithelium was 0.86 +/- 0.18 (SEM, n=72), the mean for PIN was 3.6 +/- 0.9 (n=65) and 5.8 +/- 0.4 (n=56). The difference in staining score between normal and both PIN and cancer was highly statistically significant (P<0.001, Mann-Whitney, Rank Sum test). Western blot of the cancer cell lines with anti-GGAP2 antibody reveals easily detectable protein expression in all six prostate cell lines tested with higher expression of GGAP2 in prostate cancer cell lines than in immortalized normal prostate epithelial (PNT1A) cells (Fig. 1G). Thus GGAP2 is expressed at increased levels in human prostate cancers.
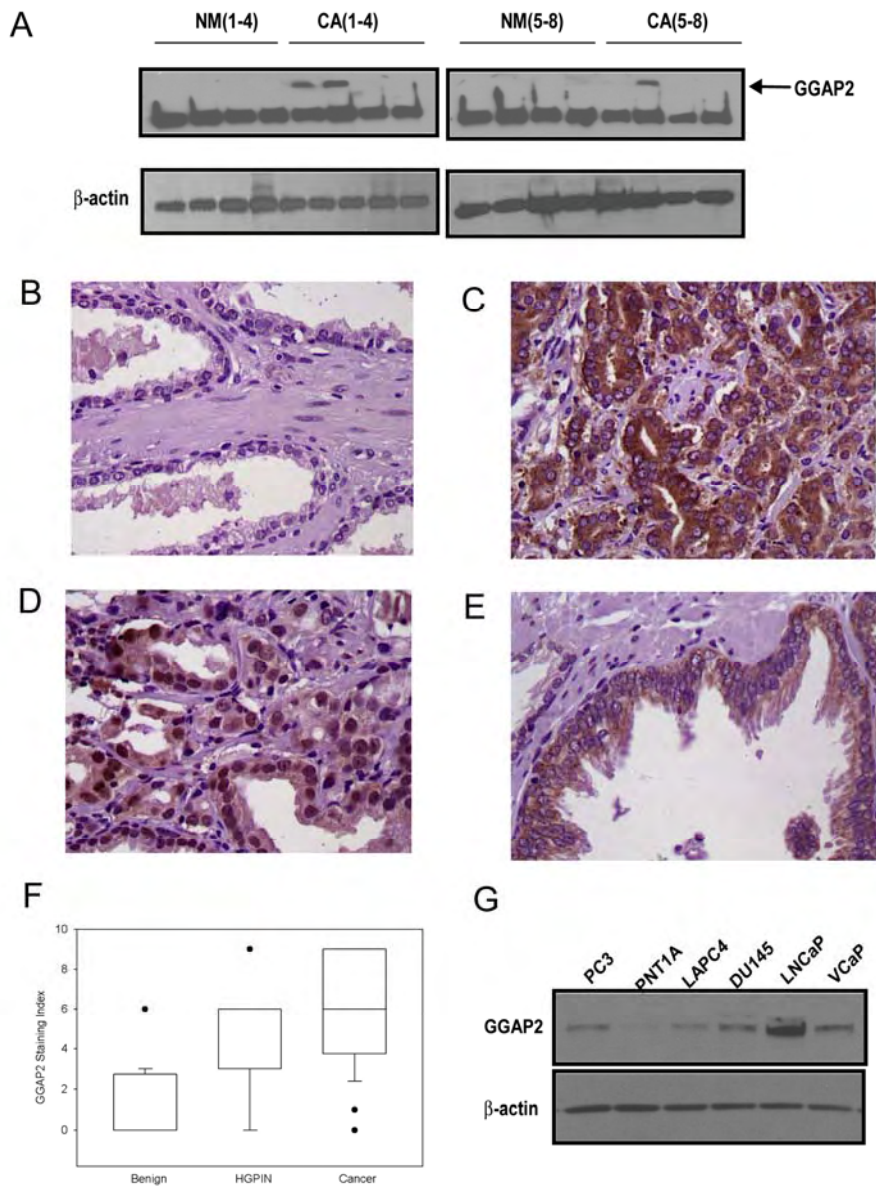
Figure 1 GGAP2 expression is increased in human prostate cancer. (A) GGAP2 is increased in clinical patient prostate tissue lysates. Lysates of 1.5 mg of total protein were used to incubate with anti-GGAP2 rabbit antibody and protein A agarose beads for 4 hours before the immunoprecipitations were immunoblotted for the same antibody. The arrow refers to the location of GGAP2 protein bands. The lower bands in each lane are antibody heavy chain. (B-E) Immunohistochemistry data showed an increased GGAP2 level in human prostate cancer tissue. Brown staining indicates GGAP2 expression. (F) Boxplot of staining indices of benign, high grade prostatic intraepithelial neoplasia (HGPIN) and cancer tissues. (G) GGAP2 was detected in prostate cancer cell lines. 20 ug of total cell lysates were used each lane. Anti-GGAP2 antibody concentration is 1ug/ml. GGAP2 was weakly detected in PNT1A cells. IP refers to immunoprecipitation and WB to Western Blotting.

Studies are now underway to carry out clinical and biological correlations using larger tissue microarrays.

Task 2: (Months 1-18) Identification of GGAP2 mutants in prostate cancers and correlation with clinical and pathological parameters of outcome

We performed RT-PCR, cloning and direct sequencing on >100 normal and cancer tissue samples to examine the GGAP2 mutation rate in prostate cancer tissue samples. Two sets of primers were used to examine the N-terminal GTPase domain and the GAP domain of GGAP2, respectively. All clones with mutations were resequenced to minimize the misreading errors in sequencing process. As shown in Table 1, although present at low frequency, multiple mutations were found in both domains in prostate cancer patient samples. Most of these mutations were detected in 1-2 out of 10-15 clones examined. Mutations in both domains of GGAP2 were detected at a significantly higher frequency in cancer tissues compared to normal. In addition, silent mutations were rare. This strongly indicates that these mutations are biologically relevant and not random mutations or PCR artifacts. This is supported by the functional characterization of these mutants (see below). Thus GGAP2 mutations are common but appear to occur later in the neoplastic progression process and so are not present within all tumor cells.

Table 1 GGAP2 mutations in human prostate cancer

	GTPase domain		
	Normal	**Late PSA recurrence**	**Early PSA recurrence**
total # of samples examined	10	10	10
total # of clones examined	55	55	55
total # of readable clones	40	37	39
total # of clones w/ mutations	1	18	12
total # of clones w/ silent mutations	2	0	0
	GAP domain		
	Normal	**Cancer**	
total # of samples examined	30	72	
total # of clones examined	320	764	
total # of readable clones	216	520	
total # of clones w/ mutations	2	195	
total # of clones w/ silent mutations	8	4	

There does not appear to be a correlation of GGAP2 mutation with patient outcome based on our data to date.

Task 3. (Months 6-30) Biochemical and biological characterization of GGAP2 mutants

To investigate the role of the identified GGAP2 mutations in prostate cancer, we used site-directed mutagenesis to clone these mutations into eukaryotic (CMV-Tag2B with a G418 selection marker, Stratagene) and prokaryotic expression vectors (GEX4T-1 for GST fusion protein purification, Promega). Stable prostate cell lines expressing control vector, wild-type GGAP2 and these mutant GGAP2 were generated using stable selection with Geneticin at recommended concentration. As Fig. 2 shows, almost all of the GGAP2 mutants stimulate PNT1A and DU145 cell proliferation to agreater extent than wild-type GGAP2 (G2), indicating that these mutations may contribute to prostate cancer growth.
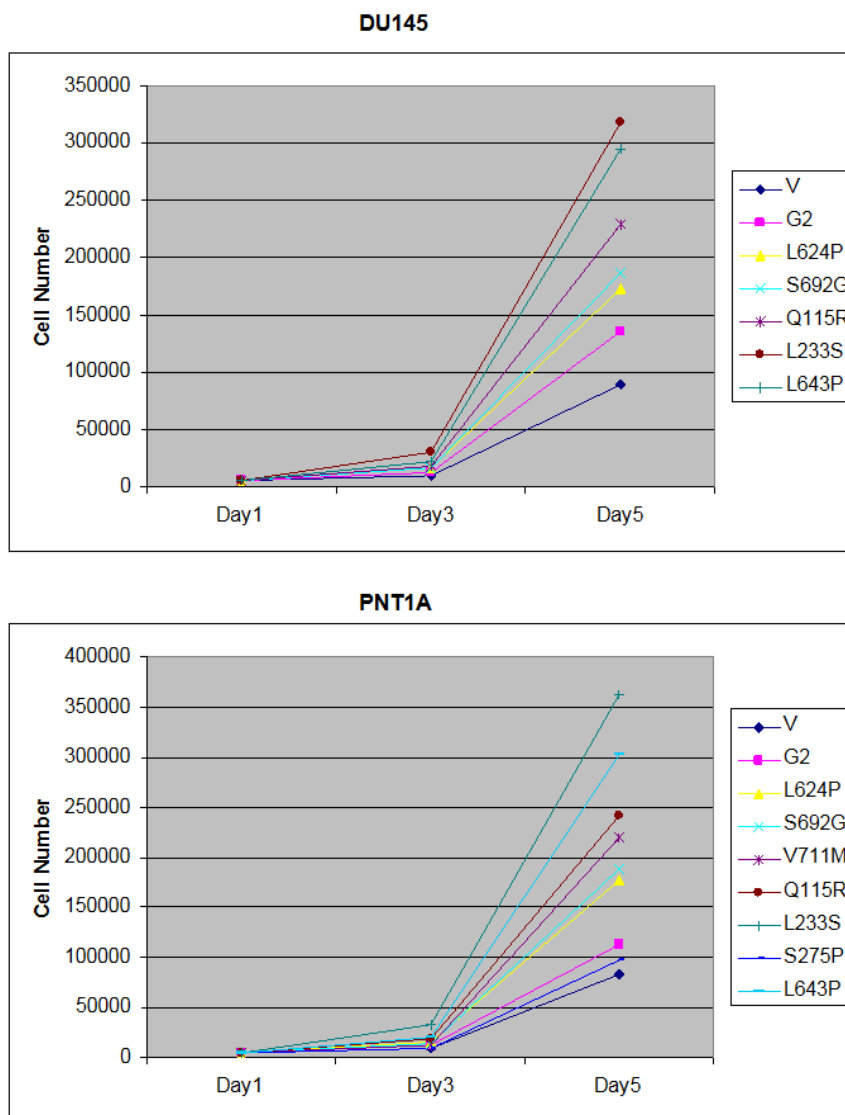

Fig 2. Proliferation of prostate cancer cells (DU145) and immortalized normal prostate epithelium (PNT1A) expressing wild-type (G2) or various mutant GGAP2 genes or vector only (V).

We also performed in vitro guanine nucleotide binding assay to investigate the effect of these clinically identified mutations on GGAP2 GTP/GDP exchange process. As shown in Figure 3, that mutant GGAP2 binds to GTP faster than wild-type GGAP2 and empty GST control beads, indicating these mutations may enhance GGAP2 activation.

3H-GDP labeling assay

Fig 3. GTP exchange in vitro of wild-type GGAP2 and mutant GGAP2. The mutant GGAP2 proteins binds to GTP faster than wild-type GGAP2 and empty GST control beads, indicating these mutations may enhance GGAP2 activation
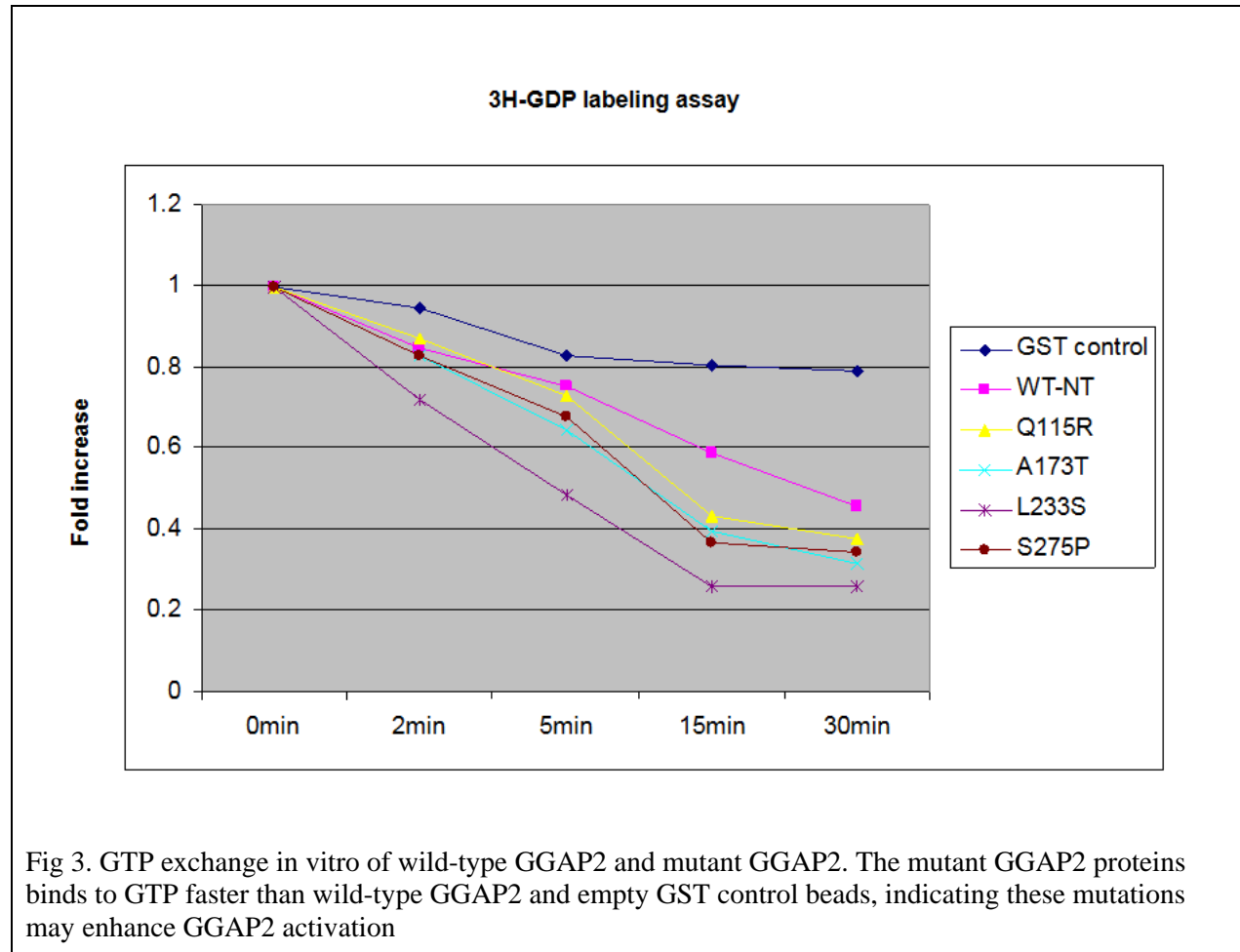
Task 4: Months (24-36) Study the effect of GGAP2 on progression and metastasis in orthotopic models of prostate cancer

We have generated PC3 and LNCAP cell lines stably expressing control vector, wild-type GGAP2, S629A (Akt phosphorylation site) mutant GGAP2 and mutant GGAP2 identified from prostate cancer tissue samples. As a preliminary experiment, 40 athymic mice were separated into two groups, and orthotopically injected with vector transfected (control) or wild-type GGAP2 transfected PC3 cells, respectively. One half of the surviving mice in each group were sacrificed at 4 weeks and the other half at 7 weeks. Tumors in the GGAP2 expressing mice were significantly larger at both 4 and 7 weeks after injection (p=0.001 and p=0.005, respectively). Histological and biological analysis of primary tumors and lymph nodes is currently in progress.

KEY RESEARCH ACCOMPLISHMENTS

- We have established that GGAP2 is expressed at increased levels in human prostate cancer
- We have shown that low frequency GGAP2 mutations are common in human prostate cancer and that they can promote biological processes that are associated with cancer progression. This implies intratumoral heterogeneity of GGAP2 mutation. This finding has important general implications for the study of mutations in prostate cancer.
- We have established key reagents and a baseline protocol for future studies of the role of GGAP2 in prostate cancer progression in vivo

REPORTABLE OUTCOMES

- GGAP2 expression is increased in human prostate cancer. These studies, along with biochemical studies of GGAP2 activities, will be submitted to *Cancer Cell* in May 2008.
- GGAP2 is mutated at high rates in human prostate cancer. We plan to submit these finding for publication in late summer or early fall 2008

CONCLUSION

GGAP2 expression is increased in prostate cancer. Our results indicate that GGAP2 is also mutated in human prostate cancer. The multiplicity of GGAP2 mutations imply that these mutations may emerge in an environment of the multifocal heterogeneity of prostate cancer tissues. According to our results these mutations can stimulate cancer cell proliferation and enhance GGAP2 activation, indicating that they may contribute to the functions of GGAP2 in prostate cancer. In summary, GGAP2 may promote prostate cancer growth and progression via overexpression and/or mutation and as such is an important therapeutic target.

REFERENCES

Xia C., Ma W., Stafford L.J., Liu C., Gong L., Martin J.F. and Liu M. (2003). GGAPs, a new family of bifunctional GTP-binding and GTPase-activating proteins. Mol. Cell. Biol. 23:2476-88.

Ahn J.Y., Rong R., Kroll T.G., Van Meir E.G., Snyder S.H., Ye K. (2004). PIKE (phosphatidylinositol 3-kinase enhancer)-A GTPase stimulates Akt activity and mediates cellular invasion. J. Biol. Chem.279:16441-51.

Agoulnik, I., Vaid, A., Nakka, M., Alvarado, M., Bingman W., Erdem, H., Frolov, A., Smith, C., Ayala, G., Ittmann, M., Weigel, N. (2006). Increased Expression of the Androgen Receptor Coactivator TIF2 is Associated with Biochemical Recurrence and Androgen Independence in Prostate Cancer. Cancer Res. 66:10594-602.